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(54) Human IFN-beta2/IL-6, its purification and uses.

(57) Monoclonal antibodies to IFN-β2/IL-6 and hybridomas producing them and production and purification of glycosylated and unglycosylated IFN-β2/ IL-6 are disclosed. The protein is useful in the treatment of breast cancer, leukemia, infectious diseases and bone marrow progenitor cell disorders.

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CONSTRUCTION OF PLASMID
pRIβ_2 604 and pRIβ_2 802

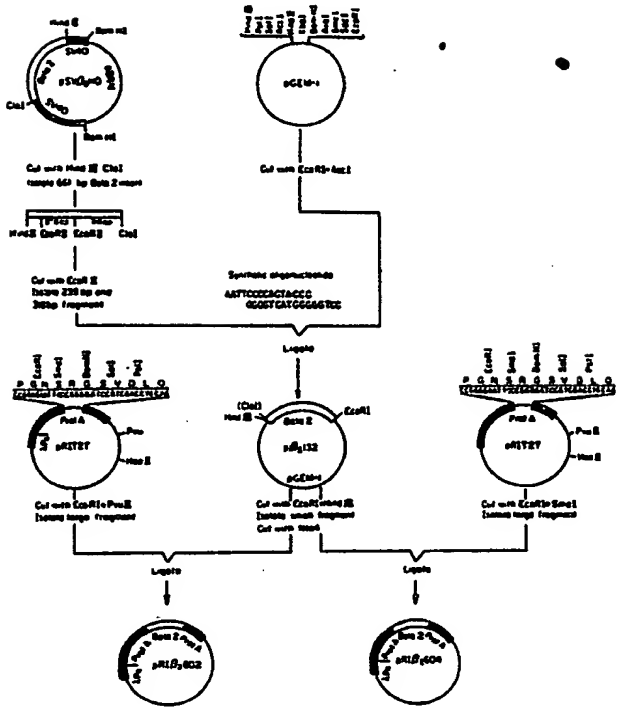


FIG. 3

Human IFN-β2/IL-6, its purificati n and uses.

FIELD OF THE INVENTION

The present invention relates to human interferon-β2 (IFN-β2), to monoclonal antibodies and to hybridoma cell lines producing said monoclonal antibodies and to a method for purification of interferon-β2 employing said monoclonal antibodies. It further relates to pharmaceutical compositions comprising human interferon-β2.

BACKGROUND OF THE INVENTION

Human interferon-β2 was first described and cloned from double-stranded RNA-induced human fibroblasts as a β-type interferon-like activity in U.K. Patent 2063882 of filing date 19.11.80 and the recombinant protein expressed by CHO cells was described in European Patent Application 220,574 of 10.10.86, both of the same applicant.

This cytokine has multiple functions and activates and it has been shown to be identical to several proteins described later on in the literature and identified by other biological activities, namely B-cell differentiation or stimulatory factor (BCDF or BSF-2), also named interleukin-6 (IL-6) (T. Hirano et al. (1986) Nature 324, pp. 73-76), hybridoma/plasmacytoma growth factor (HGF or HPGF) (J. Van Damme et al. (1987) J.Exp.Med. 165, pp. 914-919), hepatocyte stimulatory factor (HSF) (J. Gauldie et al. (1987) Proc.Natl.Acad.Sci.U.S.A. 84, pp. 7251-7255), 26-kDa protein inducible in human fibroblast cells (Haegeman et al. (1986) Eur.J.Biochem. 159, pp. 625-632), and a monocyte-derived human B-cell growth factor that stimulates growth of Epstein-Barr Virus (EBV)-transformed human B-cells (G. Tosato et al. (1988) Science 239, pp. 502-504). The protein will be designated hereinafter in this application as IFN-β2/IL-6 or IFN-β2.

Natural and recombinant IFN-β2 produced by mammalian, e.g. CHO cells appear in several forms modified by various glycosylation and phosphorylation processes (A. Zilberstein et al. (1986) EMBO J. 5, pp. 2529-2537; L.T. May et al. (1988) J.Biol.Chem. 263, pp. 7760-7768; L.T. May et al. (1988) Biophys.Biochem.Res.Commun. 152, pp. 1144-1148). Thus immunoblots of denaturating gels may show bands of about 23, 26, 45 and 66 Kd forms. The removal of the carbohydrate moiety of the glycosylated protein yields a smaller form of about 20 Kd which still retains most or all of its biological activity.

SUMMARY OF THE INVENTION

The present invention provides monoclonal antibodies capable of specifically binding to IFN-β2/IL-6 from different sources, namely to human natural IFN-β2 and to recombinant IFN-β2 expressed by mammalian, e.g. CHO or bacterial, e.g. E. coli cells.

The invention is directed also to hybridoma cell lines which produce said monoclonal antibodies.

The invention also relates to a process for the purification of human IFN-β2 which includes immunopurification.

The invention further relates to an unglycosylated polypeptide comprising the amino acid sequence of mature human IFN-β2, to recombinant vectors comprising a DNA sequence which codes for said polypeptide and microorganisms transformed therewith and to a process for producing said unglycosylated polypeptide by culturing said transformed microorganisms and recovering the polypeptide.

The invention is further directed to pharmaceutical compositions comprising IFN-β2 for the treatment of breast cancer, leukemia, infectious diseases and bone marrow progenitor cell disorders.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 shows the nucleotide sequence and the amino acid sequence of IFN-β2.
- Figure 2 shows the construction of plasmid pSVβ$_2$HB.
- Figure 3 shows the construction of plasmids pRIβ$_2$802 and pRIβ$_2$604.
- Figure 4 illustrates the analysis of IFN-β2 preparations by Western blotting.
- Figure 5 shows silver stain analysis of SDS-PAGE of IFN-β2 eluted fractions.
- Figure 6 shows the construction of plasmid pSVβ$_2$29.

- Figure 7 shows th HGF activity of unbound fractions containing IFN-β2 produced by CHO clone A2-5-10 aft r affinity chromatography with monoclonal antibody 34-1.

- Figure 8 shows th HGF activity of elution fractions containing IFN-β2 produced by CHO clone A2-5-10 after affinity chromatography with monoclonal antibody 34-1.

- Figure 9 illustrates the construction of plasmids pTLβ2501 and βKKβ27.

- Figure 10 shows silver stain analysis of SDS-PAGE of E. coli IFN-β2 after immun affinity purification and chromatography with S-Sepharose.

- Figure 11 shows inhibition of breast carcinoma cell line T47D colony formation by E. coli IFN-β2.

- Figure 12 shows results of breast carcinoma T47D and MCF-7 cells clonogenic assay with E. coli IFN-β2.

- Figure 13 shows differentiation of myeloleukemic M1 cells induced by E. coli IFN-β2.

- Figure 14 shows growth and (2′-5′) Oligo A synthetase induction in myeloleukemic M1 cells treated by IFN-β2 (in HGF units/ml).

- Figure 15 shows effect of IFN-β2 on growth of hematopoietic colonies from normal human bone marrow.

DETAILED DESCRIPTION OF THE INVENTION

The anti-IFN-β2 monoclonal antibodies of the invention are produced from a hybridoma cell line obtained by fusion of murine myeloma cells with spleen cells from a mouse previously immunized with IFN-β2 or with a fusion protein comprising IFN-β2, e.g. Protein A-IFN-β2 fusion protein.

According to the invention, there is described the construction of a plasmid for the expression in E. coli of a Protein A-IFN-β2 fusion protein and its use for obtaining monoclonal antibodies. The complete translated sequence of the cDNA coding for human IFN-β2 of Fig. 1 was fused, in phase, to the 3′ end of the coding sequence for the staphylococcal Protein A affinity tail (Uhlen et al. (1984) J.Biol.Chem. 259, p. 1695). For efficient expression in E. coli the hybrid gene was fused to the strong lambda P_R promoter.

The resulting Protein A-IFN-β2 fusion protein was purified and used to immunize mice. After six injections of the purified protein into mice, positive sera were tested for their binding titer in a solid phase radio-immunoassay (SRIA) and for the specificity of binding by Western blots. Spleen cells derived from a mouse showing the highest binding titer (dilution 1:25,000) were fused to mouse myeloma cells. The fusion of the cells is done in the presence of a suitable fusion promoter of those known in the art. The fused cells are then cultured in separate wells. The supernatant of each well is then tested for the presence of the desired monoclonal antibody capable of specifically binding to IFN-β2, preferably with IFN-β2 from a different source than the IFN-β2 used for the immunization of the mice. Thus, if a Protein A-IFN-β2 fusion protein expressed by E. coli cells is used to immunize the mice, then the screening of the monoclonal antibodies is performed with IFN-β2 produced by CHO cells. This prevents cross-reaction of the Protein A and of any E. coli contaminants in the antigen preparations used for injection with some of the monoclonal antibodies during the screening. For the SRIA, crude supernatants of CHO cells, harboring a plasmid containing the human IFN-β2 gene under the control of the SV40 early promoter and expressing high levels of this gene but no Protein A or any bacterial antigen, were bound to a solid support and reacted with supernatants of the hybridomas and with [^{125}I] goat antimouse antibodies.

The hybridomas were screened by the SRIA and several positive clones were isolated and characterized. The positive clones producing the desired antibodies are then selected and subcloned and either cultured in a suitable growth medium or injected into mice, and the desired monoclonal antibodies are then recovered from the supernatant of the cultured cells or from the ascitic fluid of said mice, respectively.

The monoclonal antibody obtained is bound to a solid phase support contained within a column. Any suitable gel matrix known in the art may be used to immobilize the monoclonal antibody e.g. agarose-polyacryl-hydrazide. Crude preparations of human IFN-β2 preparations are loaded on the column and IFN-β2 is eluted from the gel by a change in pH or ionic strength. In a preferred embodiment, the fractions containing IFN-β2 are eluted with a buffer pH 2 and neutralized immediately after elution with a buffer pH 8.5.

The crude natural IFN-β2 preparations purified according to the invention are obtained from induced fibroblast cells together with IFN-β1.

The recombinant IFN-β2 produced by CHO cells to be purified is obtained according to the process described in A. Zilberstein et al. (1986) EMBO J. 5, pp. 2529-2537.

According to the present invention, an unglycosylated polypeptide comprising the amino acid sequence of IFN-β2 is obtained by recombinant DNA techniques. Human DNA, particularly cDNA coding for a

polypeptide comprising the amino acid sequence for IFN-β2, is fused through the coding region to a strong bacterial promoter, such as hybrid tryp-lac promoter, and this fused DNA molecule is inserted into a suitable plasmid so as to obtain a recombinant vector comprising said DNA sequence and regulatory regions which ar positioned in such a way that expression of said polyp ptide in bacterial cells is possibl . Bacterial cells, e.g. E. coli, are transformed by said recombinant plasmids and cultured in order to express th desired polypeptide, which is subsequently recovered and purified.

The IFN-β2 molecule as described is a polypeptide comprising 212 amino acids. At the N-terminus of the protein may be found the sequences Ala^{28}-Pro-Val-Pro-Pro- or -Pro^{29}-Val-Pro-Pro- or -Val^{30}-Pro-Pro- of Fig. 1. In our preferred embodiment, the IFN-β2 cDNA was fused at the Pro^{29} codon to a Met initiator codon and a tryp-lac promoter, but this and all other sequences are covered by the present invention as long as the polypeptide has IFN-β2/IL-6 activity.

The DNA vectors used in this invention were constructed by standard procedures. Plasmid DNAs were purified by banding in CsCl-ethidium bromide gradients. DNA restriction fragments separated by electrophoresis in agarose or polyacrylamide gels were purified on DE-52 columns. Restriction endonucleases (Boehringer, New England Biolabs), T4 DNA ligase (New England Biolabs), the large fragment of E. coli DNA polymerase (Boehringer) and T4 polynucleotide kinase (Pharmacia), were used as recommended by the suppliers. E. coli transformation was carried out with frozen competent bacteria (D.A. Morrison (1979) Methods Enzymol. 79, pp. 326-331) using strains HB101 ATCC 33694, JM101 ATCC 33876, N4830-1 (Gootesman et al. (1980) J.Mol.Biol. 140, p. 57) and JM105 (Messing et al. (1981) Nucleic Acids Res. 9, pp. 309-321).

The present invention discloses the hematopoietic effect of IFN-β2 and its use in the inhibition of breast carcinoma cell growth, in the growth inhibition and differentiation of myeloleukemic cells and in the induction of Complement Factor B in fibroblasts. Thus human IFN-β2 is used as active ingredi nt of pharmaceutical compositions for the treatment of breast cancer, leukemia, infectious diseases and bone marrow progenitor cell disorders.

The invention will now be illustrated by the following examples, without delimiting its scope:

Example 1. Preparation of Protein A-IFN-β2 fusion protein

A) Construction of plasmids pRI$\beta_2$802 and pRI$\beta_2$604

Plasmid pSVβ_2HB (Fig. 2) is one of the vectors used for constitutive expression of the IFN-β2 gene in CHO cells under the strong SV40 early promoter and is derived from plasmid pSVCIFβ_2 (A. Zilberstein et al. (1986) EMBO J. 5, pp. 2529-2537) by removing all the 5′ and 3′ non-coding sequences of the cDNA by standard cloning techniques.

The 661 bp cDNA insert coding for IFN-β2 was excised from plasmid pSVβ_2HB as a 661 bp Hind III/Cla I fragment and digested with Eco RII. The resulting five fragments were separated on a preparative agarose gel. The three small fragments of 55, 12 and 37 bp coding for the signal peptide sequence and for the first three amino acids of the mature protein were discarded and the two fragments of 239 and 318 bp w r recovered from the gel. In order to restore the sequence coding for the first amino acids and to maintain the Protein A frame, a double stranded synthetic oligonucleotide was prepared (sequence shown in Figure 3) and ligated together with the 239 bp and 318 bp fragments into plasmid pGEM-1 previously digested with Eco RI and Acc I. The resulting plasmid was called p$\beta_2$132 (Fig. 3) and contains the whole IFN-β2 sequence preceded by an asparagine and a serine codon within the multiple coding site of plasmid pGEM-1. The asparagine and serine codons are the two codons at the unique Eco RI site (at the 3′ end of the Protein A gene) of plasmid pRIT2T (Pharmacia) that was used for subsequent cloning. Plasmid p$\beta_2$132 was digested with Eco RI and Hind III and the complete IFN-β2 cDNA sequence was isolated and introduced into plasmid pRIT2T digested either with Eco RI and Pvu II or with Eco RI and Sma I restriction endonucleases for the obtention of the plasmids pRI$\beta_2$802 and pRI$\beta_2$604, respectively (Fig. 3).

B) Production of Protein A-IFN-β2 fusion protein and its purification

Strain E. coli N4830-1 (Gootesman et al. (1980) J.Mol.Biol. 140, p. 57) was transformed with recombinant plasmids pRI$\beta_2$802 and pRI$\beta_2$604 giving origin to new microorganisms E. coli N4830-1/pR$\beta_2$802 and E. coli N4830-1/ pR$\beta_2$604, respectively.

Diluted cell cultures of the microorganisms were grown overnight at 30° C in M9 medium containing ampicillin to early stationary phase, then incubated at 42° C for 90 minutes, cooled and harvested by centrifugation. After repeated resuspension and centrifugation, 20% SDS was added to a final concentration of 1% and 10M urea to a final concentration of 8M, and th extract containing the expressed Protein A-IFN-β2 fusion protein was dialyzed against TST (50 mM Tris pH 7.6, 150 mM NaCl and 0.05% Tween 20). The clear supernatant after dialysis was applied to the IgG Sepharose 6 Fast Flow (FF) equilibrated column. After loading on the column the gel was washed and the bound fusion protein was eluted with 0.5 M NH_4COOH, pH 3.4 and lyophilized directly without prior dialysis.

Example 2. Preparation of anti-IFN-β2 monoclonal antibodies

A) Immunization of mice and cell fusion

Three-month old female Balb/c mice were first injected with the partially purified Protein A - IFN-β2 fusion protein obtained in Example 1 above (10μg/mouse, emulsified in complete Freund's adjuvant). Three weeks later the mice were given a subcutaneous boost with the fusion protein in solution. Four additional injections were given at 10 days intervals. The mouse showing the highest binding titer (Table 1) and the strongest signal in Western blot analysis received an intra- peritoneal injection of the fusion protein and three days later its splenic lymphocytes (150×10^6 cells) were fused with 30×10^6 NSO/I myeloma cell line. The fused cells were distributed into microculture plates (3×10^4 cells/well) and selected for hybridoma growth. Hybridomas that were found to secrete anti-IFN-β2 antibodies were clones and recloned by the limiting dilution technique.

B) Screening for anti-IFN-β2 monoclonal antibody-producing specific hybridomas

Hybridoma supernatants were tested for the presence of anti-IFN-β2 antibodies by a solid phase radioimmunoassay (SRIA). PVC microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with a crude, serum-free supernatant of CHO cells secreting IFN-β2 (80μg/well). Following an incubation of 2 hrs at 37° C or 16 hrs at 4° C the plates were washed twice with PBS containing BSA (0.5%) and Tween 20 (0.05%) and blocked in washing solution for 2 hrs at 37° C. Hybridoma culture supernatants (50 μ/well) were added and the plates were incubated for 4 hrs at 37° C. The plates were then washed three times with the washing solution and ^{125}I-goat anti-mouse $(Fab')_2$ (50μl, 10^5 cpm) was added for further incubation of 16 hrs at 4° C. The plates were washed 4 times and individual wells were cut and counted in a gamma counter. Samples giving counts that were at least four times higher than the negative control value were considered positive (Table 1).

Table 1

Screening of hybridomas by SRIA		
Sampl	dilution	cpm
Immune serum (mouse)	1:4000	2800
negative control (mouse)	1:4000	100
Hybridoma 12		4000
12	1:125	300
27		1100
28		2200
34		6200
34-1	1:2500	1000
38		2600
48		1500
102		1200
117		1400
123		1100
125		1600
132		5400
136		2700
154		1500
157		2400
negative hybridoma		200
Ascitic fluid	1:62,000	1400
Ascitic fluid negative	1:12,000	300

As can be seen from Table 1, fourteen anti-IFN-β2 hybridomas were selected using the SRIA. Hybridoma No. 34-1, sub-cloned from hybridoma 34, was further characterized and was found to belong to IgGI class. Hybridoma 34-1 was deposited with the Collection Nationale des Cultures de Microorganismes - CNCM, Institute Pasteur, Paris, on 14.11.88. It was accorded No. I-813.

Hybridoma 34-1 was found suitable for Western blotting and for affinity purification of natural and recombinant IFN-β2 expressed both by E. coli and by CHO cells. It was used in the following experiments.

Example 3. Applications of the anti-IFN-β2 monoclonal antibodies

A) Western blotting

Samples of crude preparations of either natural or recombinant IFN-β2 expressed by CHO and E. coli cells were analyzed by SDS-PAGE under reducing conditions and electroblotted onto nitrocellulose sheets (BA85, Schleicher and Shuell). Following electroblotting the sheet was incubated overnight with a blocking buffer (5% non-fat milk in PBS containing 0.05% Tween 20 and 0.02% sodium azide) and then for 2 hrs at room temperature with the anti-IFN-β2 antibody No. 34-1. Following washing in 0.05% Tween 20 in PBS, the nitrocellulose was incubated for 3 hrs at room temperature with ^{125}I-goat anti-mouse serum (0.7×10^6 cpm/ml in the blocking buffer). The sheet was then washed, dried and autoradiographed. The results are shown in Fig. 4: Lane A: natural IFN-β2; Lane B: recombinant IFN-β2 of CHO cells; Lane C: recombinant IFN-β2 of E. coli cells; Lane D: recombinant IFN-β1 of CHO cells (comparison).

B) Affinity chromatography of IFN-β2 preparations

Ascitic fluids of mice containing monoclonal antibodies secreted by hybridoma 34-1 were precipitated with ammonium sulfate (50% saturation) 16 hrs at 4° C. The precipitate was collected by centrifugation, redisolved in water and dialysed against saline. About 10 mg of immunoglobulins were bound to 1 ml agarose-polyacryl-hydrazide according to Wilcheck and Miron ((1974) Methods Enzym. 34, p.72). Crude

preparations of either natural (fibroblast) or recombinant (E. coli or CHO) IFN-β2 (containing 0.5 M NaCl) were loaded at 4° C at a flow rate of 0.25 ml/min. The column was washed with 30 column volumes of 0.5 M NaCl in PBS. IFN-β2 was eluted by 50 mM citric acid buffer, pH 2 (8 x 1 column volume fractions) and immediately neutralized by 0.1 M Hepes buffer, pH 8.5.

Crude recombinant IFN-β2 (E. Coli extract depleted from DNA) was loaded on 1 ml of the anti-IFN-β2 column. Purification of 1000 fold was achieved in one step, and the recovery of IFN-β2 was 100% (Tabl 2). The procedure was scaled by using 8 ml affinity column. The capacity of the column was 400 µg pur IFN-β2 per 1 ml of column. Silver stain analysis of SDS-PAGE of the eluted fractions revealed a major band of a M.W. of 21,000 and some minor contaminants of a higher M.W. (Fig. 5). When crude recombinant IFN-β2 (CHO) was loaded on 1 ml affinity column, purification was achieved in one step with a recovery of 100%. Silver stain analysis of SDS-PAGE of the eluted fractions revealed two major bands of 23 kDa and 28 kDa, both belonging to the glycosylated forms of IFN-β2 (Fig. 5). The same bands were obtained when natural IFN-β2 (foreskin fibroblasts) was immunoaffinity purified.

Table 2

Source of IFN-β2	Sample	HGF units/ml x 10^{-4}	Prot.conc. mg/ml	Spec.act. units/mg	Purif. fold	Recovery %
E. coli	load	1.4	4.1	3400		
	effluent	0.54	4.1	1300		
	eluate	25	0.09	3.6×10^6	1060	100
CHO	load	0.2				
	effluent	0.06				
	eluate	2.7	0.14	0.2×10^6		100
Foreskin fibroblast	load	0.014	1.46	95		
	effluent	0.014	-			
	eluate	0.014	0.008	17×10^4	180	100

Example 4. Monitoring of IFN-β2 produced by CHO cells

IFN-β2 produced and secreted in one liter of culture medium by CHO clones was quantitated using monoclonal antibody 34-1 for purification by immunoaffinity.

Clone A2-5-10 is a CHO clone obtained by transfection of CHO cells with plasmid pSV$\beta_2$29 (Fig. 6) and selection with 50 nM methotrexate (MTX).

Plasmid pSV$\beta_2$29 was obtained as follows: a DNA fragment containing the sequence coding for IFN-β2 fused to the early promoter of SV40 and to the SV40 polyadenilation site was excised from plasmid pSVc15 as a 2.5 kb BamHI fragment. Plasmid pSVc15, one of the vectors previously used for constitutiv expression of IFN-β2 in CHO cells under the strong SV40 early promoter, was derived from plasmid pSVCIFβ_2 (A. Zilberstein et al. (1986) EMBO J. 5, pp. 2529-2537) by removing all the sequence 5' to th XhoI site of the IFN-β2 cDNA. This BamHI fragment was cloned into the BamHI site of a pDHFR plasmid containing a mouse DHFR cDNA fused to the SV40 early promoter and a splicing region of mouse IgG gamma-2a.

The clone A2-5-10 was grown to confluency in roller bottles. The culture medium was changed to a low (2%) fetal calf serum and collected 24 hours after the change. One liter of culture was concentrated to 45 ml and loaded on the monoclonal antibody affinity column prepared in Example 3B. The column was extensively washed and the bound IFN-β2 was eluted with 50 mM citric acid pH 2, in four fractions of on ml each. The IFN-β2 purified in this way seems to be homogeneously pure as indicated by silver stain analysis of SDS-PAGE (Figure 5). The amount of IFN-β2 protein recovered from one liter of culture was 469µg.

The amount of IFN-β2 in each fraction was estimated by measuring the hybridoma growth factor (HGF) activity of the protein in the crude preparation and in the different fractions of the affinity column. About 40% of the IFN-β2 loaded on th column was recovered in the unbound fraction (Figure 7), while the remaining activity was recovered in fractions eluted with pH 2 (Figur 8) with a peak in elution 2. These results indicate that, under the conditions described abov , clone A2-5-10 produces about 800 µg/l of IFN-

β2.

The specific acitivity of the IFN-β2 produced and secreted by the CHO clone A2-5-10 was determined by measuring the HGF activity and the protein concentration in each of the purified fractions of the immuno-affinity column. On unit of HGF is defined as the amount of protein that gives 50% of the maximal effect in th assay. Th HGF activity was assayed in 0.1 ml cultures of murin plasmacytoma T1165 cells, treated for 24 hours and pulsed for 16 hours with [^{3}H]thymidine as described by Nordan R.P. and Potter M. (1986) Science 233, pp. 566-568. Tabl 3 summarizes th r sults of such an analysis. Th specific HGF activity of IFN-β2 in the three fractions, eluted from the affinity chromatography column, ranged from $1.18x10^6$ to $2.1x10^6$ with an average of $1.47x10^6$.

Table 3

Specific Activity of IFN-β2			
Fraction	HGF activity U/ml	Protein concentration mg/ml	Specific activity U/ml
Elution 1	128,000	0.108	$1.18x10^6$
Elution 2	333,000	0.239	$1.39x10^6$
Elution 3	166,000	0.079	$2.10x10^6$
Total	627,000	0.426	$1.47x10^6$

The elution fractions from the affinity chromatography column were pooled, dialyzed to 10mM acetate buffer pH 5 and loaded on a Mono S cation exchange column (Pharmacia). The column was washed with 10 mM acetate buffer pH 5 and then eluted using a linear sodium chloride gradient from 0 to 600 mM. HGF activity was determined in the different fractions. Activity coincided with the main peak of protein.

Example 5. Preparation and purification of recombinant IFN-β2 produced by E. coli cells

A) Construction of plasmids pTLβ2501 and pKKβ27

Plasmid pβ2324 containing the whole IFN-β2 sequence preceded by an ATG codon within the multiple coding site of plasmid pGEM-1 ws prepared in the same way as plasmid pβ2132 in Example 1A and Figure 3, except for the fact that a different synthetic oligonucleotide (Fig. 9) was used. Plasmid pβ2324 was digested with Eco RI and Hind III and the complete IFN-β2 cDNA sequence was isolated and introduced into either one of plasmids pTLα143-4 (Y. Chernajovsky et al., (1983) Ann. N.Y. Acad. Sci. 413, pp. 88-96) or pKK223-3 (Pharmacia) digested with Eco RI and Hind III for the obtention of the plasmids pTLβ2501 and pKKβ27 (Fig. 9).

B) Expression of the biologically active IFN-β2 polypeptide in E. Coli

Strain E. coli JM105 (described in J. Messing et al., (1981) Nucleic Acids Res. 9, pp. 309-321) was transformed with recombinant plasmid pKKβ27 giving origin to new microorganism E. coli JM105/pKKβ27 deposited with the ATCC under the Budapest Treaty on 17.12.1987 and assigned the number ATCC 67583. The transformation of strain E. coli JM101 (ATCC 33876) with the recombinant plasmid pTLβ2501 gave origin to new microorganism E. coli JM101/pTLβ2501 deposited with the ATCC under the Budapest Treaty on 17.12.1987 and assigned the number ATCC 67584. The microorganisms were culture, lysed and the extracts containing IFN-β2 were purified.

C) Purification of E. coli IFN-β2 by chromatography

Nucleic acid-free bacterial extracts were obtained by polyethyleneimin precipitation and passage on DEAE cellulose. The effluent fractions were adsorbed onto S-Sepharose in 10 mM Na acetate buffer pH 5

(buffer A) and eluted with a 0-0.03 M NaCl gradient. Active fractions were pooled, dialyzed against buffer A, chromatographed on a Mono-S FPLC column and eluted with a 0-0.03 M NaCl gradient. HGF activity was followed during purification and coincided with the IFN-β2 protein revealed by immunoblots using polyclonal antibodies against a N-terminus peptide of IFN-β2. The final preparation showed a single band of about 20 kDa on SDS-polyacrylamide gel electrophoresis.

D) Immunopurification of E. coli IFN-β2

For purification, 1-1.5 l of Dyno-mill extract obtained from 3-6 liter of fermentor culture were precipitated with polyethyleneimine, the solution was concentrated down to 100 ml (A4) or 500 ml (A8) and loaded on an 8 ml column of monoclonal antibody 34-1 (Ig from ascites, 8 mg Ig/ml column) in phosphate buffered saline (PBS) with 0.5M NaCl pH 7.0 (A4) or 1M NaCl (A8). After washing the column with the same buffer, elution was carried out by 50mM citric acid buffer pH 2 (A4) or with the same citric buffer and propylene glycol 25% (A8) and samples were immediately neutralized by addition of 0.1M Hepes buffer pH8.5. The results were as follows:

A4: Immunopurification of E. coli IFN-β2:					
Fraction	Volume	Protein	HGF activity	HGF U/ml	HGF U/mg
Load eluted:	90 ml	5,700 mg	29 million U	325,000	5,100
Total	40 ml	8.2 mg	2.9 million U		
(Tube 3)	10 ml	2.2 mg		110,000	500,000

A8: Immunopurification of E. coli IFN-β2:					
Fraction	Volume	Protein	HGF activity	HGF U/ml	HGF U/mg
Load eluted:	350 ml	2,550 mg	10 million U	330,000	4,100
Total	37 ml	5.2 mg	22 million U		4.2 million
(Tube 3)	9.5 ml	2 mg		1.3 million	

Following immunoaffinity, an S-Sepharose column was used as the final purification step. In a typical experiment, (S12), the input from pooled A4 fractions was dialyzed against 10mM acetate buffer pH 5, adsorbed and eluted by a gradient from 0.1-0.4 M NaCl. The peak eluted at 0.3 M. The results were as follows:

S12: S-Sepharose after immunoaffinity					
Fraction	Volume	Protein	HGF activity	HGF U/ml	HGF U/mg
Input:					
A4 pool	17.5 ml	3.6 mg	1.4 million U		0.5 million
pH5 dial.	22.5 ml	2.2 mg	ND		
Eluted:					
Total peak	4.5 ml	0.6 mg	0.9 million U		2 million
Tube 58	0.5 ml	0.04mg		210,000	5.2 million

The yield in this step was 64%. The product was run on SDS polyacrylamide gel under reducing and non-reducing conditions and showed one singl band at 21 Kd. (Fig. 10).

Example 6. Growth inhibition f breast carcinoma cells

While IFN-β2/IL-6 stimulates growth of plasmacytoma/hybridoma cells (HGF activity), it is growth

inhibitory on other cell types. We hav studied colony formation by the T47D line of human Breast Ductal carcinoma cells (ATCC HTB 133), which is poorly inhibited by IFN-β1 but is sensitive to inhibition by IFN-β2. Fig. 11 shows inhibition of T47D colony formation in culture dishes by pure E. coli rIFN-β2/IL-6. The 50% inhibition is seen with 10-20 HGF U/ml. At doses over 100 U/ml, the remnant colonies ar very small and appear to represent mainly growth arrested cells (insert). Inhibition of ^{3}H-thymidine incorporation in semiconfluent T47D cells by E.coli and CHO rIFN-β2/IL-6 was found to be neutralized by th anti-IFN-β2 monoclonal antibody 34-1.

To investigate if the inhibition of ^{3}H-thymidine incorporation in T47D cells represents a g nuine antigrowth activity, we used a 15 day clonogenic assay (Fig. 12). A 50% decrease in the number of colonies of T47D cells was observed with 2 BSF-2 U/ml of E. coli IFN-β_2 and an almost complete inhibition of growth in these conditions was reached at 50 U/ml. The mock preparation had no inhibitory activity in this clonogenic assay (Fig. 12 a). In the same experiment we observed that IFN-β_1 at 500 antiviral U/ml produced no inhibition of T47D cell growth. Decreased colony formation was similarly observed in another breast carcinoma cell line MCF-7 (ATCC HTB 22) (Fig. 12 b). By ^{3}H-thymidine incorporation, MCF-7 appears somewhat less sensitive than T47D cells.

For the clonogenic assay, T47D cells were seeded at 200 cells per well and MCF-7 at 1,000 cells per well in 6-well Costar plates in 1 ml RPMI 1640 with 10% fetal calf serum (FCS), insulin 0.2 U/ml, glutamine 2 mM, penicillin 100 U/ml, streptomycin 100 ug/ml. Cells received 24 hours later serial 5 fold dilutions of E. coli IFN-β2 or mock E. coli preparations at the same protein concentration. After 15 days the colonies were stained with crystal violet and counted under an inverted microscope. For DNA synthesis measurements, cells were seeded at 15-25 x 10^3 per well of a 96-well microplate, and after 3 days FCS was removed for 24 hours and readded in fresh medium with serial 5 fold dilutions of E. coli IFN-β2 or mock preparations. After 16-24 hours, cells were labeled with 15 μCi/ml ^{3}H-thymidine (25 Ci/mmol, Amersham) for 1 hour, washed twice with PBS, treated by 5% trichloroacetic acid (TCA) for 30 min at 4° C and washed 3 times with 5% TCA. The precipitate was dissolved in 0.1 ml of 0.2 M NaOH at 37° C for 30 minutes, neutralized by 0.01 ml of 2M HCl and counted in a Tricarb counter with toluene scintillator and Lumax (3:2 v/v). Similar results were obtained without serum starvation, ^{3}H-thymidine incorporation was lower but inhibition was the same.

Extracts containing IFN-β2 were assayed for BSF-2 activity as measured by the stimulation of IgG secretion by CESS cells in response to treatment with said extracts (A. Muraguchi et al., (1981) J. Immunol. 128, pp. 1296-1301; T. Hirano et al., (1985) Proc. Natl. Acad, Sci. USA82, pp. 5490-5494) and for HGF activity as measured by the ability of the extracts to support the growth of plasmacytoma cell line T1165 (R.P. Nordan and M. Potter, (1986) Science 233, pp. 566-569). Stimulation of ^{3}H-thymidine incorporatio in T1165 cells and of IgG secretion by CESS cells showed half-maximum at a dilution of 1:12,500 which was therefore defined as one unit of BSF-2/HGF activity. This unitage is used in the present experiments.

Example 7. Growth inhibition and differentiation of myeloleukemic cells

IFN-β2 is also active in growth inhibition and differentiation of myeloleukemic cells. Murine myeloleukemic Ml cells and human histiocytic lymphoma U937 cells were grown in RPMI 1640 with 10% fetal calf serum (FCS). The cells were seeded at 10^5 per ml in wells of 12-well Costar plates. Pure E. coli IFN-β2 was added at 0.1-75 ng/ml and the cultures were observed for 4-6 days. Cells were counted and stained for Giemsa and for non-specific esterase using the α-naphtyl acetate esterase kit 91-A of Sigma (St. Louis, MO). Lysozyme activity was measured in 0.5% Triton-X100 cell extracts by a turbimetric assay of the lysis of Micrococcus lysodeikticus (Sigma Co.), the assay being calibrated with egg-white lysozyme as described (Weisinger, G. and Sachs, L. (1983) EMBO J. 3, pp. 2103-2107). The (2'-5') oligo-A synthetase activity was assayed in Nonidet-P40 cell extracts as described (Resnitzky et al. (1986) Cell46, pp. 31-40).

Table 4

Effect of IFN-β2/IL-6 on myeloleukemic Ml cell growth					
IFN-β2 BSF U/ml	Cell number x 10^{-5}				
	Day 0	Day 1	Day 4	Day 5	Day 6
0	1	2.3	19.0	25.0	35.0
25	1	2.0	3.5	0.9	0.7
50	1	1.4	2.3	0.8	1.2
Recombinant E. coli IFN-β2 purified by Mono-S FPLC (Example 5C)					

Without addition, the M1 cells grew without adhering to the dish and showed typical myeloblastic morphology. In contrast, after 4 days of culture with IFN-β2, the cells were adherent and showed dramatic morphological changes (Figure 13). About 60% of the cells acquired macrophage-like morphology, the rest showing various degrees of maturation. Cytoplasms were enlarged, contained vacuoles and acquired typical foamy appearance. Nuclei were eccentric, less round and contrasted and had less prominent nucleoli. Viable cell counting showed that while the control culture grew for 6 days, the M1 cells treated by 50 U/ml IFN-β2 underwent 2-3 divisions and growth was arrested (Table 4). At day 4 after seeding, less than 1 U/ml IFN-β2 (expressed in plasmacytoma growth units) was sufficient to cause a 50% decrease in M1 cell number. The growth-arrest effect was maximal about 30U/ml IFN-β2 (15 ng/ml). Even with the chemically purified rIFN-β2, this concentration corresponds to no more than 2.5 pg/ml LPS which had no effect on the M1 cells. Growth inhibition and differentiation of M1 cells was observed when IFN-β2 was added with 5 µg/ml polymyxin B, further excluding any role of LPS traces. As a biochemical marker of differentiation we measured lysozyme activity in extracts of 5 x 10^6 M1 cells cultured 4 days with 30 U/ml IFN-β2. Lysozyme was undetectable in the control M1 cultures. Treatment with IFN-β2 induced lysozyme to levels of 0.85 µg lysozyme equivalent per 5 x 10^6 cells. Phagocytic activity on latex beads was also observed in the differentiated M1 cells.

In another experiment, addition of IFN-β2/IL-6 to cultures of M1 cells arrested the growth of the cells after 24 hours (Fig. 14) and induced their differentiation into macrophages. At 24 hours, the cells already showed cytoplasmic enlargement with acentric nuclei and after 3-4 days acquired typical macrophage morphology demonstrated by increase in lysozyme phagocytotic activity and increase in Mac 1 antigen. The 50% growth inhibition of M1 cells was observed with about 0.5 ng/ml of rIFN-β2/IL-6, less than what is required for stimulation of plasmacytoma T1165 cells. The effect of IFN-β2/IL-6 was more rapid than that of the combination of IL-1 (10 U/ml) and TNF (10^3 U/ml) which produced growth arrest only after 48 hours. These cytokines which also cause M1 differentiation are known inducers of IFN-β2/IL-6. The growth-arrest by IFN-β2/IL-6 was fully neutralized by monoclonal antibody 34.

Table 5

Effect of IFN-β2/IL-6 on histiocytic lymphoma U937 cell growth and differentiation			
Expt	IFN-β2 BSF U/ml	Cell number x 10^{-5}	Esterase positive Cells, per cent
1.	0	14.0 (100).0	4
	100	10.0 (71)	24
2.	0	26.7 (100)	N.D.
	150	23.5 (88)	"
	1500	14.5 (54)	"
Cells treated for 5 days with or without rIFN-β2 purified on Mono-S FPLC. (Example 5C)			

Human histiocytic lymphoma U937 cells can be induced to differentiate by phorbol esters and Vitamin D3, partially by IFN-gamma and other yet unidentified cytokines. We examined the effect of 100 HGF U/ml IFN-β2 addition on U937 cultures. After 4-5 days, about 25% of the cells showed monocytic/macrophage morphology and ther was a 30% reduction in cell growth (Table 5). The cells wer stained for α-naphtyl acetate esterase as a biochemical marker of differentiation not induced by IFN-gamma. About a fourth of the cells in the IFN-β2 treated culture were strongly positive for th non-specific esterase, whereas few positive cells were observed in the non-treated culture (Table 5) or in mock treated cultur (not shown). With higher amounts of the pure rIFN-β2 preparations, growth inhibition (Table 5) and partial morphological changes, such as cytoplasmic enlargement and nucleus indentation, were more pronounced. However, w found that when added together with IFN-gamma, the effect of low dose IFN-β2 was significantly potentiated (Table 6). Under these conditions, cell growth was reduced and most of the cells showed cytoplasmic enlargement, changes in nuclear shape and nucleoli reduction, although monocytic differentiation was still incomplete. Thus we found that the combination of IFN-gamma 100 U/ml and IFN-β2 (1-10 HGF U/ml) has a synergistic effect and triggers growth arrest and differentiation. In optimal conditions, IFN-gamma alone reduced growth (after 6 days) by 10%, IFN-β2 alone by 25% and the combination IFN-gamma and IFN-β2 by 90%.

Table 6

Synergistic effects of IFN-β2/IL-6 and IFN-gamma on histiocytic lymphoma U937 cells

IFN-β2 BSF U/ml	IFN-gamma U/ml	Cell number x 10^{-5}	(2′-5′) A Synthetase Activity ^{32}P-A2′pA, cpm
0	0	20 (100)	110
15	0	14 (70)	310
0	100	12 (60)	940
15	100	8.5 (42)	4,000

Cells treated for 6 days with or without rIFN-β2 immunoaffinity purified.

The addition of IFN-gamma also strongly potentiated the induction of (2′-5′) Oligo A synthetase by IFN-β2 (Table 6) suggesting that the two cytokines cooperate to initiate the differentiation process, although other additions may be required to see complete differentiation of the type seen with the M1 cells.

IFN-β2 activity on fresh leukemic cells of acute myelogenous leukemia (AML) was also studied. Peripheral blood mononuclear cells from AML patients incubated 5 days with rIFN-β2/IL-6 showed a decrease in the percentage of blast cells (from 20-30% in control cultures to 6-12% with IFN-β2/IL-6) with an increase in myelocytic forms at various stages of differentiation and in the ratio of myelomonocytes to blast cells. The results with two AML patients are shown in Table 7. GM-CSF was also tested (comparison). IFN-β2/IL-6, therefore acts also on fresh leukemic cells and such tests may be useful to foresee th therapeutic value of the cytokine in AML.

Table 7

Effect of IFN-β2/IL-6 on blood cells from AML patients

	Patient # 1		Patient # 2	
	Percent Blasts	Ratio: Myelomonocytes / Blast cells	Percent Blasts	Ratio: Myelomonocytes / Blast cells
After 5 days of culture with:				
FCS ALONE	30	2.3	20	3.6
+GM-CSF	39	1.6	40	1.4
+IFN-β2 (CHO17)	11	8.1	4	22.5
+IFN-β2 (E.COLI)	15	5.3	7	12.4

Example 8. Hematopoietic effects on normal bone marrow cultures

Monocyte and T-cell depleted human bone marrow cells treated by 4-hydroperoxy cyclophosphamide (4-HC) (100μg/ml, 30 min.) to eliminate committed progenitors of CFU-mix (colony-forming unit - granulocyte, monocyte, erythroid, megakaryocytes), CFU-GM (colony-forming unit -granulocyte, monocyte) and BFU-E (burst forming unit - erythroid) colonies, were used to study the effects of rIFN-β2/IL-6 on the early steps in hematopoietic differentiation. When added at the time cells were plated on methylcellulose (10^5 cells/ml), IFN-β2/IL-6 by itself could not support the growth of colonies, indicating it does not function as a growth-promoting CSF (colony stimulating factor) (Fig. 15). However, IFN-β2/IL-6 markedly increased the ability of IL-3 to cause formation of colonies with mixed (CFU-GEMM) and erythroid (BFU-E) as well as granulocytic monocytic (CGU-GM) phenotypes. In this action IFN-β2/IL-6 appears more potent than IL-1 (Fig. 15). In a two-stage assay, where IFN-β2/IL-6 wass added in liquid cultures one week before the cells were plated in methylcellulose with a full supplement of CSF, an increase in the number of progenitor cells able to respond to CSF was produced by IFN-β2/IL-6 alone (Fig. 15, right). This increase was only slightly lower than that caused by IL-3 and the two factors seem to work independently in this first stage of the assay. In Fig. 15, colonies were counted after 15 days and classified as CFU-mix, CFU-GM and BFU-E Left half: Day 0 cultures with no addition (10% fetal calf serum and erythropoietin), and with the addition of 10 HGF U/ml rIFN-β2/IL-6, 2 U/ml rIL-1, 10 U/ml rIL-3, IFN-β2/IL-6 + IL-3 or IL-1 + IL-3. Right half: Cells were first incubated for one week in liquid cultures with no addition and in the presence of rIFN-β2/IL-6, rIL-1. rIL-3, IFN-β2/IL-6 + IL-3 or Il-1 + IL-3. The cells were then plated as above in methylcellulose for 15 days with PHA-induced leucocyte conditioned medium (containing all CSF) and colonies counted.

The stimulation of mixed colonies from normal bone marrow progenitor cells is significant enough to warrant the use of rIFN-β2/IL-6 in bone marrow transplants.

Example 9. Induction of Complement Factor B in fibroblasts

The induction of Complement Factor B in human diploid skin fibroblasts GM8399 by IFN-β2 was studied. When used alone, the immunopurified IFN-β2 induced the secretion of Complement Factor B but the effect was again strongly potentiated by IFN-gamma. This effect is of importance since Complement Factor B is an essential component of the alternative pathway of complement which kills bacteria and parasites without need for antibodies. A local increase in resistance to such infectious agents can be expected in response to the IFN-β2 - IFN-gamma combination. A biological assay for Factor B activity showed increase in complement activity for cell lysis. The synergistic effect of IFN-gamma and IFN-β2 suggest that this combination may prove very attractive.

Pharmaceutical Compositi ns

Human IFN-β2 may be used according to the invention for the treatment of br ast cancer, leukemia, e.g. acute myelogenous leukemia, infectious diseases caused by bacteria or parasit and in bone marrow transplants. It may be used alone or in combination with other cytokines, in particular with IFN-gamma for the treatment of infectious diseases and certain types of leukemia. The active IFN-β2 may be administered by any route appropriate to the condition being treated. It may be formulated with one or more pharmaceutically acceptable carriers and systematically administered either parenterally, intravenously or subcutaneously, or enterally, e.g. in the form of a tablet, capsules, etc.

The amount of active ingredient to be administered will be determined by the physician and will depend upon several factors, e.g. the severity of the condition being treated, weight, age and general condition of the patient, the route of administration chosen and the specific activity of the active IFN-β2. Daily dosages could be in the range of about 5 micrograms to about 800 micrograms, preferably within the range of 10-100 micrograms per day.

The pharmaceutical compositions of the invention may conveniently be presented in unit dosag form and may be prepared by any of the methods well known in the art.

Claims

1. A monoclonal antibody to human interferon-β2 (IFN-β2) which specifically binds to both human natural and recombinant IFN-β2.

2. A monoclonal antibody according to claim 1 of class IgGl.

3. A hybridoma cell line which produces a monoclonal antibody according to claim 1 or 2 which is obtained by fusion of murine myeloma cells with spleen cells from a mouse previously immunized with human IFN-β2 or with a fusion protein comprising human IFN-β2.

4. The hybridoma cell line CNCM I-813 (No. 34-1).

5. A method for preparing a monoclonal antibody according to claim 1 which comprises the steps of:

a) immunizing mice with human IFN-β2 or with a fusion protein comprising human IFN-β2;

b) fusing the spleen cells from said mice with murine myeloma cells in the presence of a suitable fusion promoter;

c) culturing the fused cells and testing the supernatant of the cultures fused cells for the pres nce of the desired monoclonal antibody with human IFN-β2 from a different source than the IFN-β2 used in step (a) above;

d) selecting and cloning the hybridoma cell line producing the desired monoclonal antibody, and either

(i) culturing the selected hybridoma cell line in a suitable growth medium and recovering the desir d monoclonal antibody from the supernatant, or

(ii) injecting the selected hybridoma cell line into mice and recovering the desired monoclonal antibody from the ascitic flud of said mice.

6. A method according to claim 5 wherein in step (a) the mice are immunized with Protein A - IFN-β2 fusion protein expressed by E. coli and the hybridoma supernatants of step (c) are tested with IFN-β2 expressed by CHO cells.

7. A method for producing a monoclonal antibody according to claim 1 or 2 which comprises either culturing hybridoma cell-line CNCM I-813 in a suitable growth medium and recovering the monoclonal antibody from the supernatant of said hybridoma, or injecting hybridoma cell line CNCM I-813 into a mouse and recovering the monoclonal antibody from the ascitic fluid of said mouse.

8. An antigenic Protein A-IFN-β2 fusion protein for use in the preparation of a monoclonal antibody according to claim 1 or 2.

9. A method for the immunopurification of human IFN-β2 wherein a sample containing human IFN-β2 is passed through an immunoadsorbent column comprising a monoclonal antibody according to claim 1 or 2 bound to a solid phase support.

10. A method according to claim 9 wherein the human IFN-β2 to be purified is human recombinant IFN-β2 produced by CHO or E. coli cells, the fractions containing IFN-β2 are eluted and neutralized immediately after elution and then further purified on a Mono S cation exchange or S-Sepharose column respectively.

11. Unglycosylated polypeptide comprising the amino acid sequence of mature human interferon-β_2, being a single polypeptide of a molecular weight of about 20,000.

12. A recombinant vector comprising a DNA sequence which codes for a polypeptide comprising the amino acid sequence of mature human interferon-β_2 and regulatory regions which are positioned in a way that expression in bacterial cells of th polypeptide comprising the amino acid sequence of mature human interf ron-β_2 is possible.

13. A recombinant vector according to claim 12 wherein the vector is the plasmid pKK$\beta_2$7 or th plasmid pTL$\beta_2$501.

14. A microorganism transformed by a recombinant vector according to claim 12 or 13.

15. A microorganism according to claim 13 which is E. coli JM 105/pKK$\beta_2$7 (ATCC 67583) or E. coli JM 101/pTL$\beta_2$501 (ATCC 67584).

16. A process for producing an unglycosylated polypeptide as claimed in claim 11 which comprises culturing a transformed microorganism according to claim 14 or 15, causing the microorganism to express said polypeptide and recovering it.

17. Use of human IFN-β2 for the preparation of a pharmaceutical composition for the treatment of breast cancer, leukemia, infectious diseases or bone marrow progenitor cell disorders.

18. Use of human IFN-β2 according to claim 17 for the preparation of a pharmaceutical composition for the treatment of acute myelogenous leukemia (AML).

19. Use of human IFN-β2, optionally in association with IFN-gamma, for the preparation of a pharmaceutical composition for the treatment of infectious e.g. bacterial or parasitic diseases.

20. The use of human IFN-β2 for the treatment of breast cancer, leukemia, infectious diseases or bon marrow progenitor cell disorders.

IFN-Beta 2a cDNA

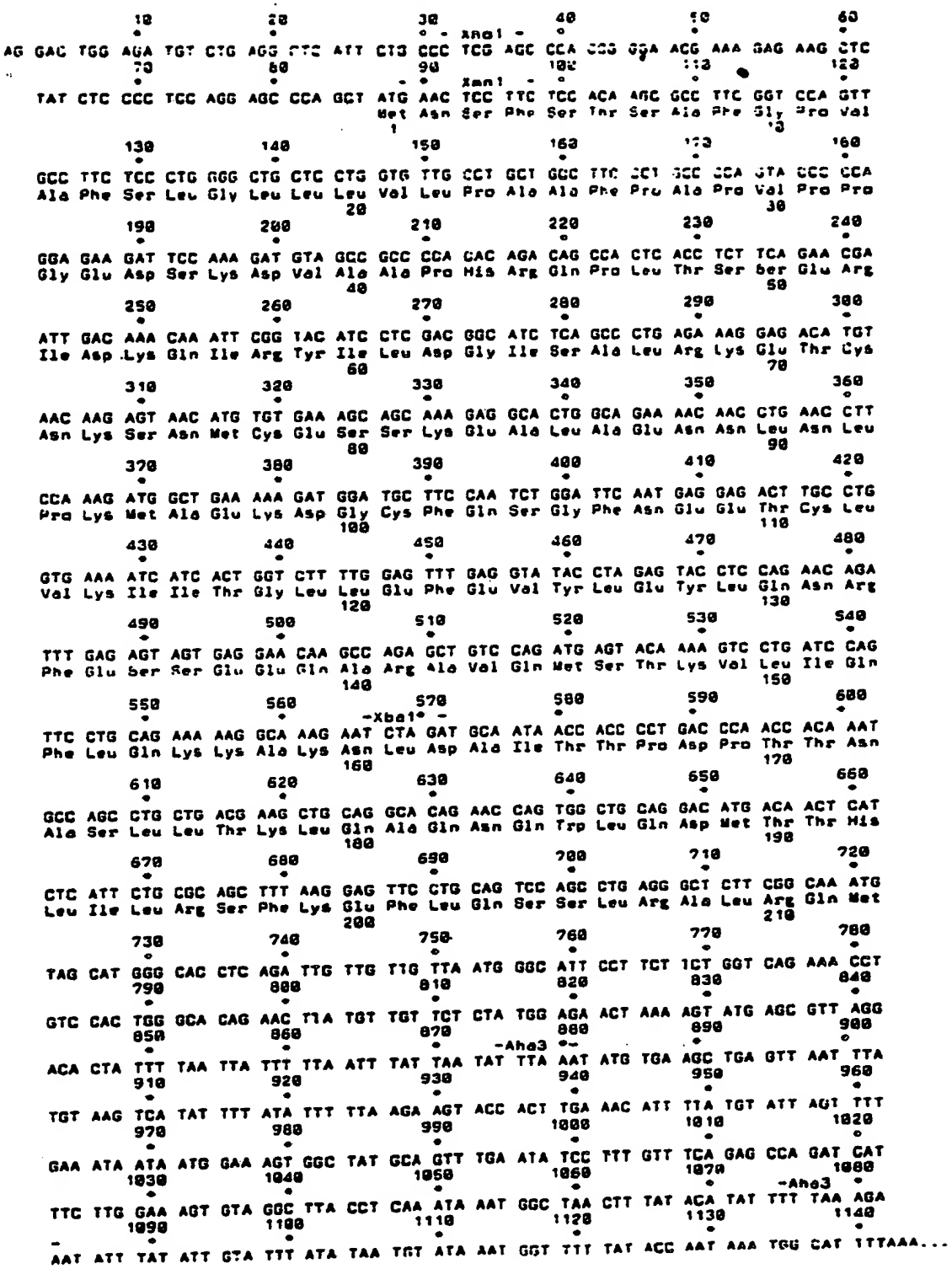


FIG. 1

CONSTRUCTION OF EXPRESSION VECTOR pSVβ_2HB

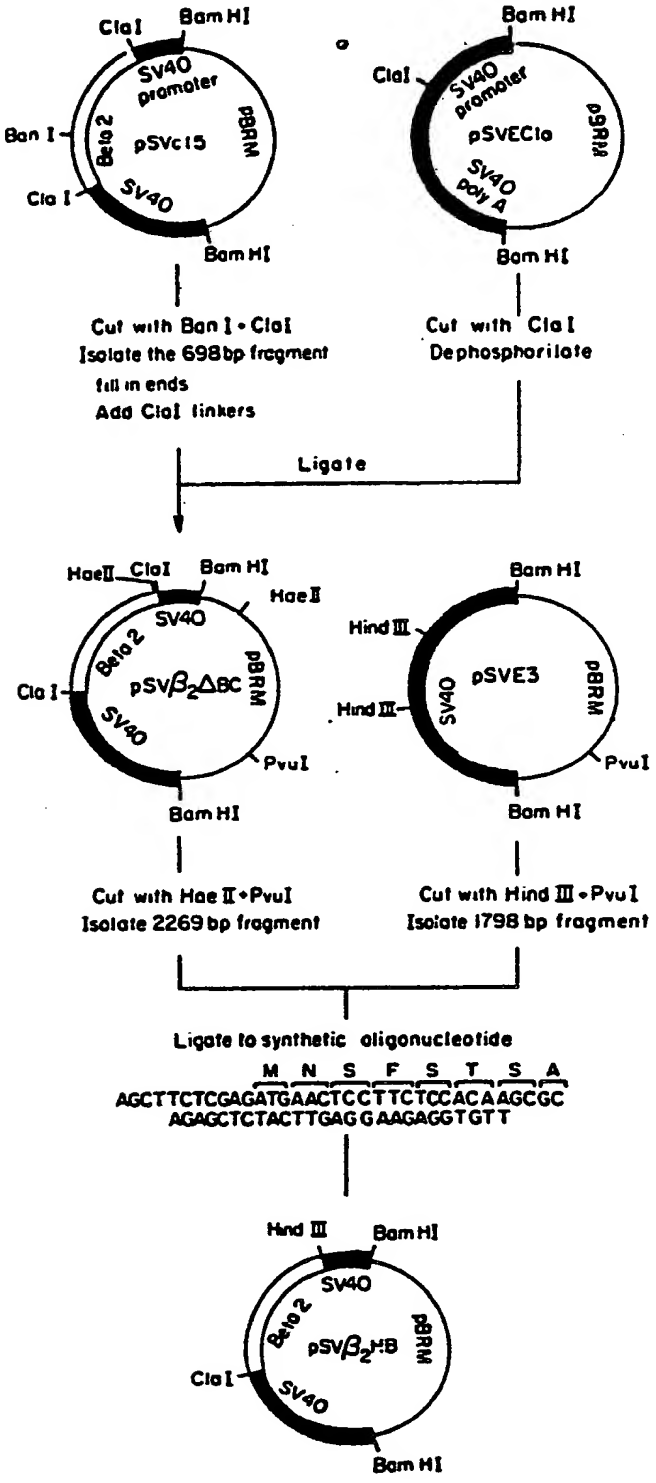


FIG. 2

CONSTRUCTION OF PLASMID pRIβ2 604 and pRIβ2 802

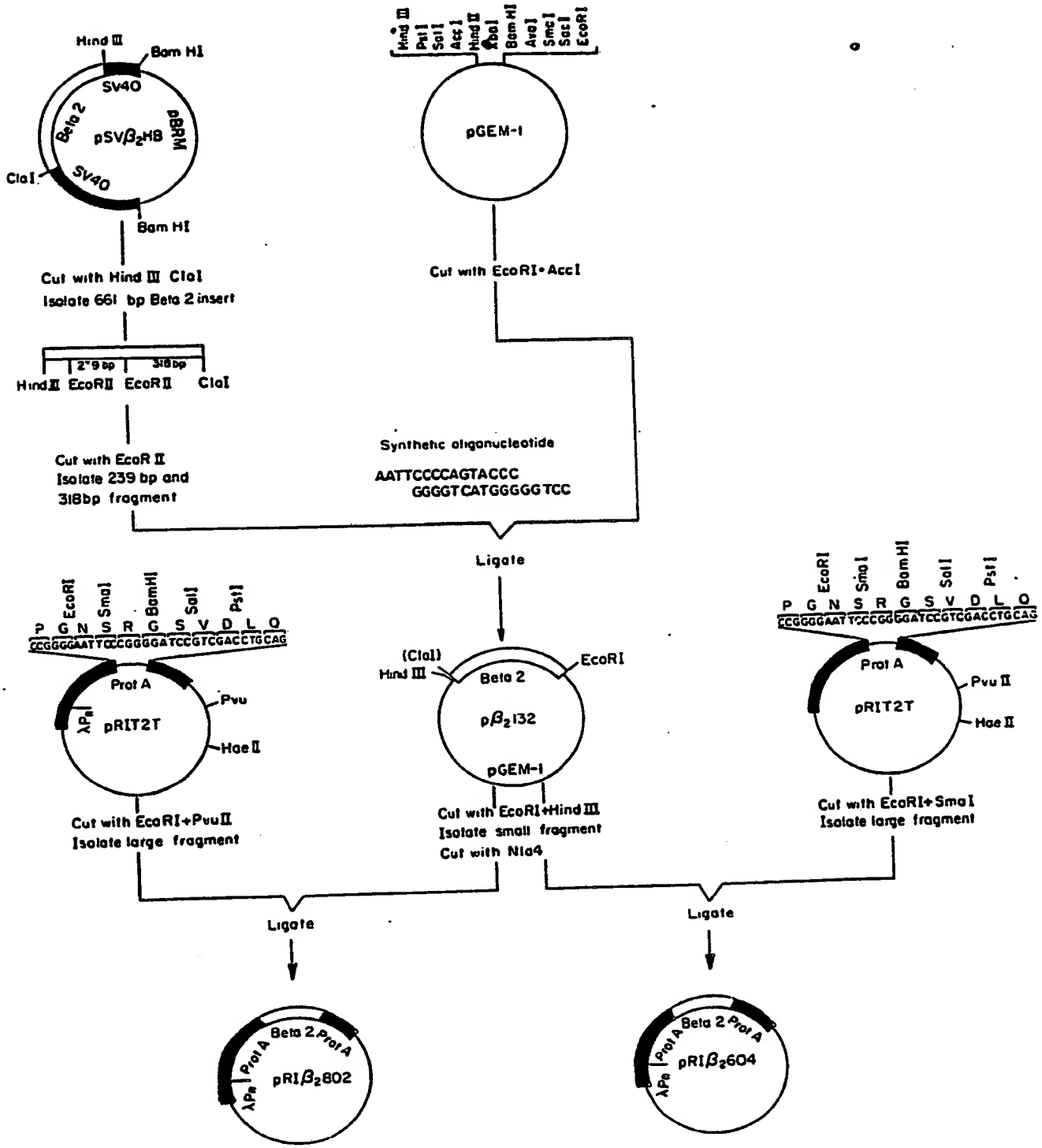


FIG. 3

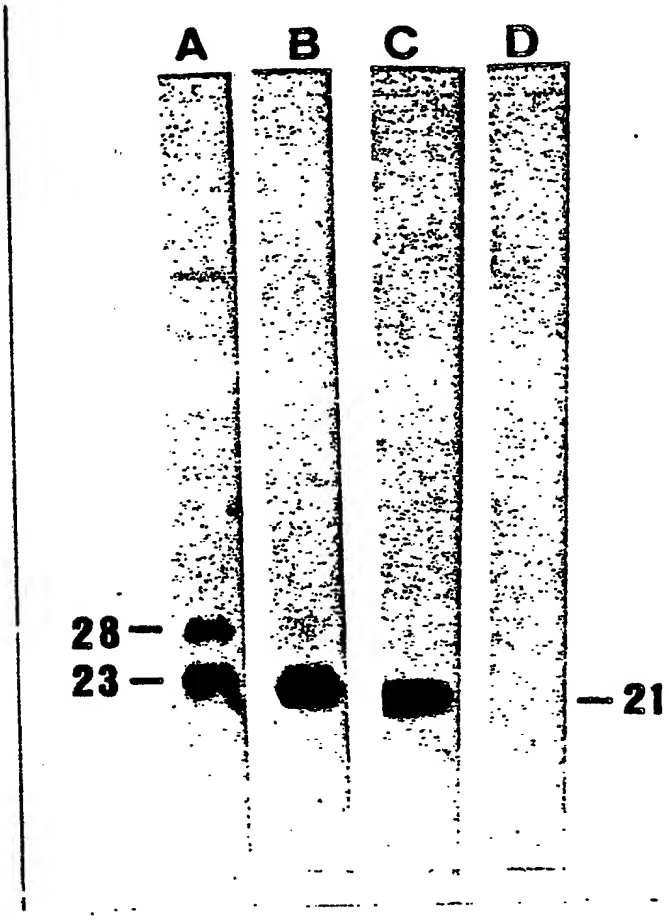


Fig. 4

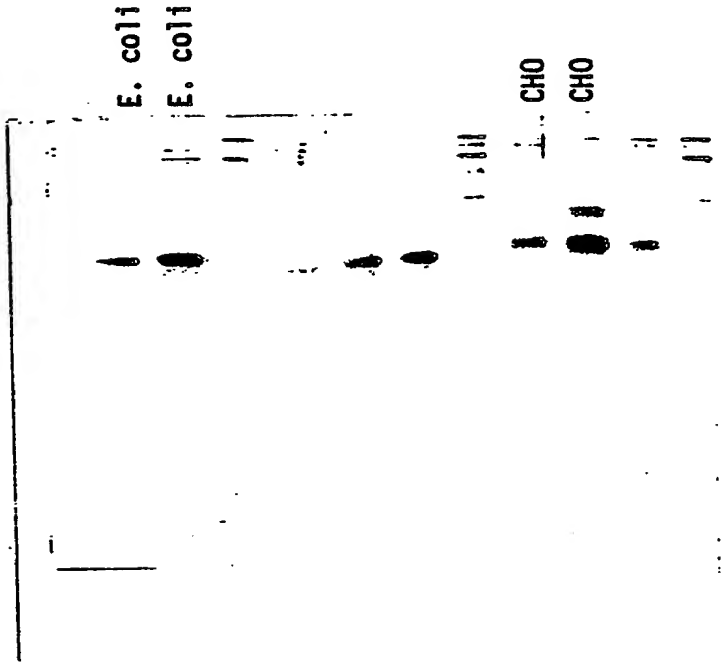


Fig. 5

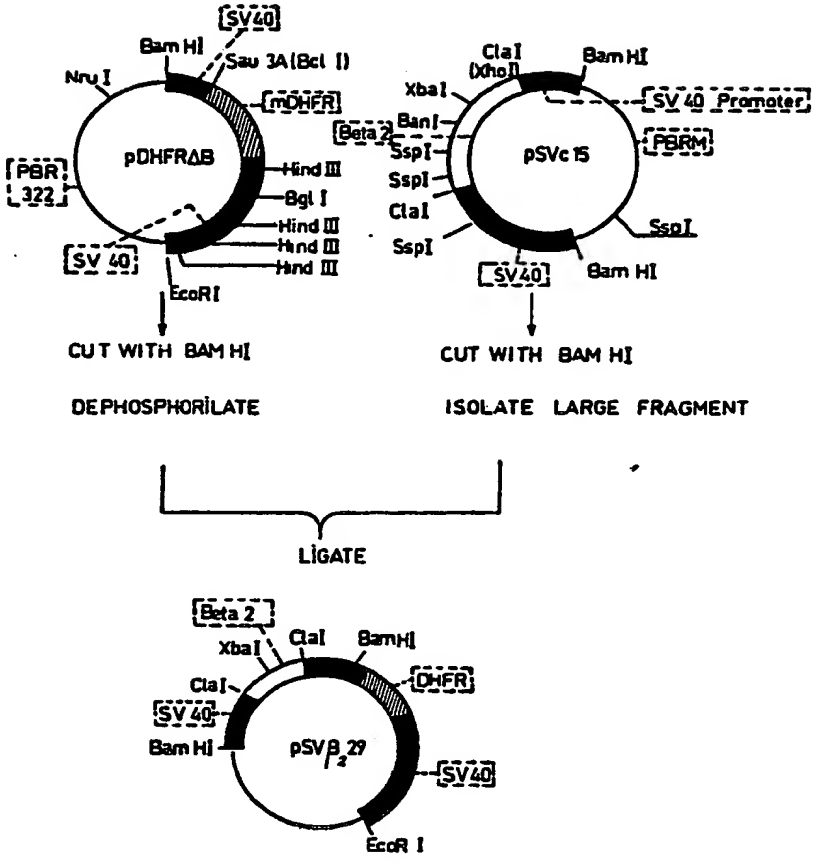
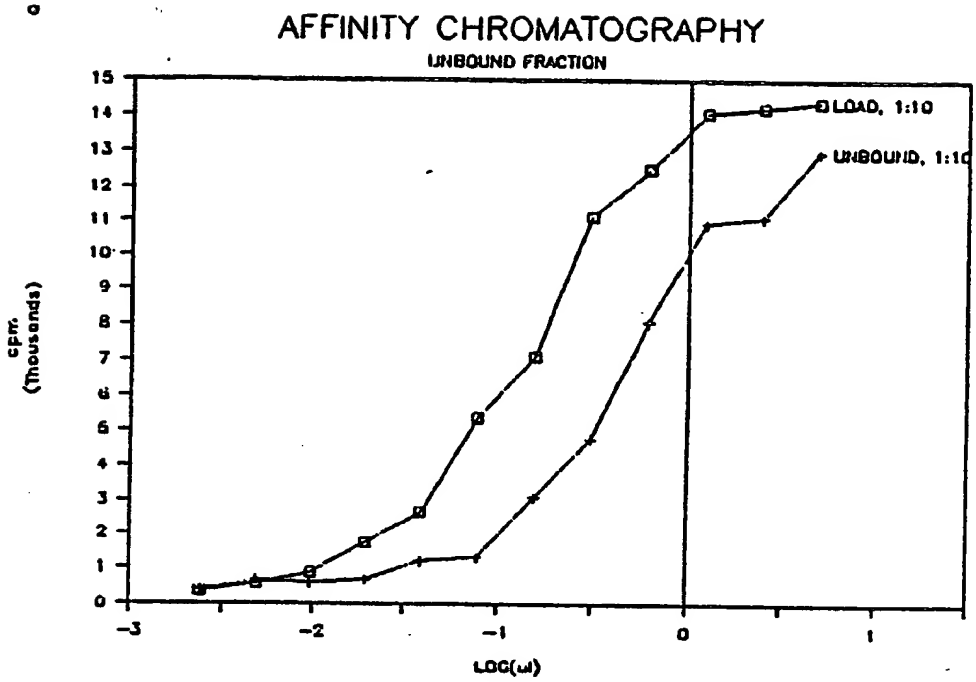

SV40
Bam HI
Sau 3A (Bcl I)
Nru I
mDHFR
pDHFRΔB
PBR 322
Hind III
Bgl I
Hind III
Hind III
Hind III
SV 40
EcoRI
CUT WITH BAM HI
DEPHOSPHORİLATE
ClaI (XhoI)
Bam HI
Xba I
SV 40 Promoter
Ban I
Beta 2
SspI
pSVc 15
PBRM
SspI
ClaI
SspI
SspI
SV40
Bam HI
CUT WITH BAM HI
ISOLATE LARGE FRAGMENT
LİGATE
Beta 2
Xba I
ClaI
BamHI
DHFR
ClaI
SV40
Bam HI
pSVβ2 29
SV40
EcoR I

FIG. 6


AFFINITY CHROMATOGRAPHY
UNBOUND FRACTION
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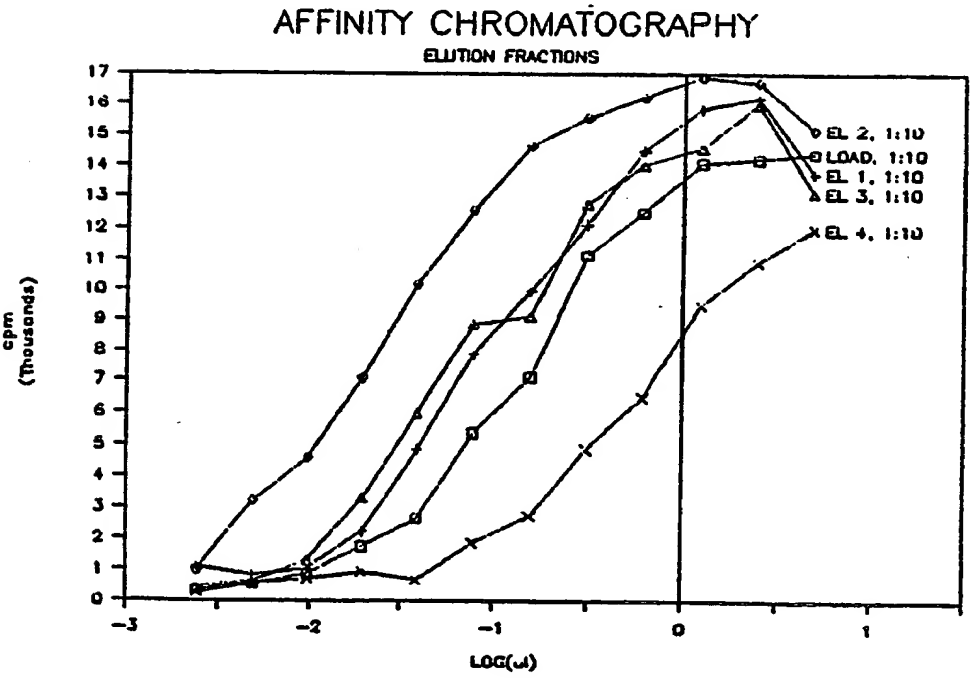
UNBOUND, 1:10
cpm (Thousands)
LOG(ul)

FIG. 7


AFFINITY CHROMATOGRAPHY
ELUTION FRACTIONS
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LOAD, 1:10
EL 1, 1:10
EL 3, 1:10
EL 4, 1:10
cpm (Thousands)
LOG(ul)

FIG. 8

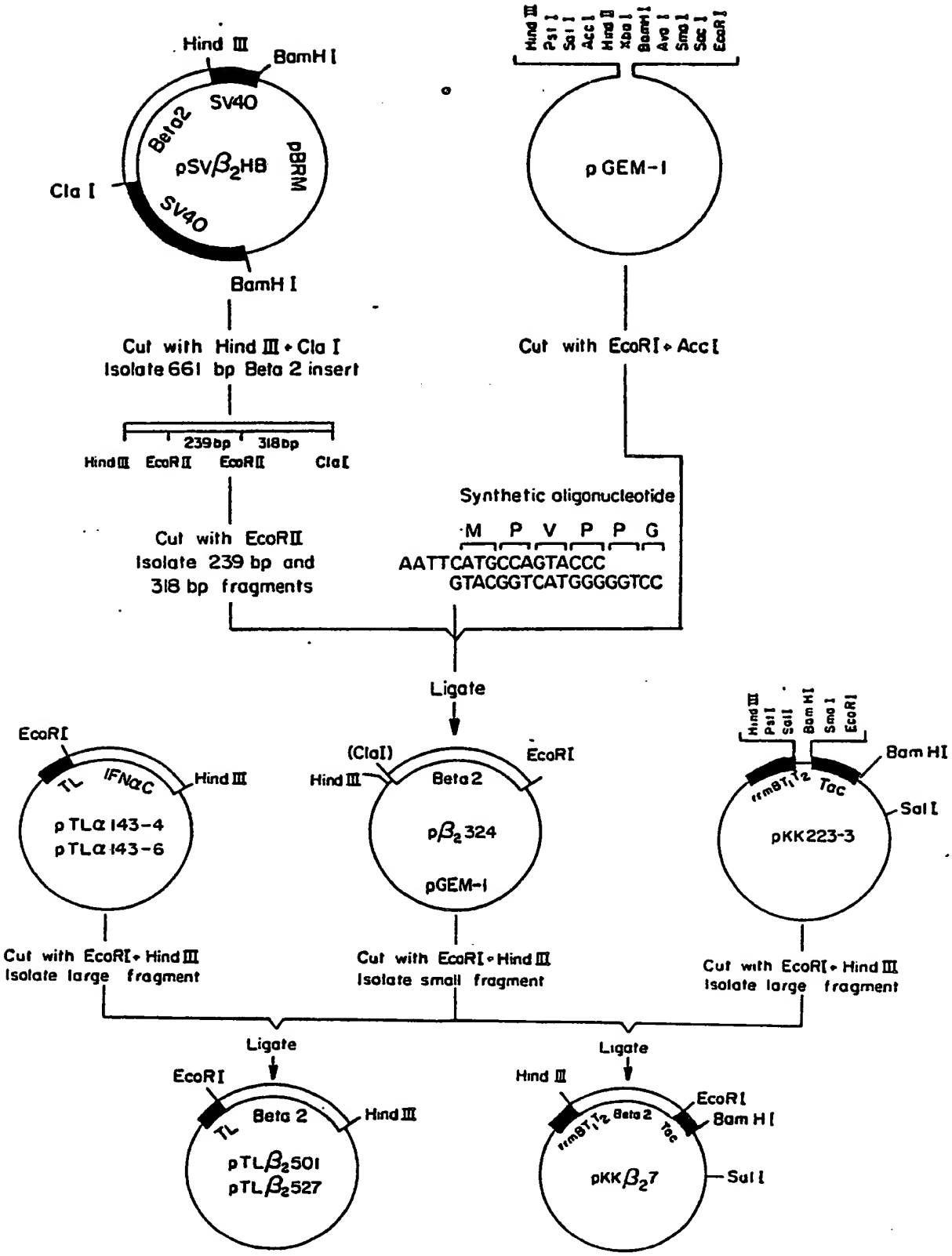
Hind III
BamH I
SV40
Beta2
pSVβ2HB
pBRM
Cla I
SV40
BamH I
Cut with Hind III + Cla I
Isolate 661 bp Beta 2 insert
239bp
318bp
Hind III
EcoR II
EcoR II
Cla I
Cut with EcoR II
Isolate 239 bp and
318 bp fragments
Hind III Psi I Sal I Acc I Hind II Xba I BamH I Ava I Sma I Sac I EcoR I
pGEM-1
Cut with EcoR I + Acc I
Synthetic oligonucleotide
M P V P P G
AATTCATGCCAGTACCC
GTACGGTCATGGGGGTCC
Ligate
EcoR I
TL
IFNαC
Hind III
pTLα143-4
pTLα143-6
Cut with EcoR I + Hind III
Isolate large fragment
(Cla I)
EcoR I
Hind III
Beta2
pβ2324
pGEM-1
Cut with EcoR I + Hind III
Isolate small fragment
Hind III Psi I Sal I Bam HI Sma I EcoR I
Bam HI
rrnBT1T2
Tac
Sal I
pKK223-3
Cut with EcoR I + Hind III
Isolate large fragment
Ligate
EcoR I
TL
Beta 2
Hind III
pTLβ2501
pTLβ2527
Ligate
Hind III
EcoR I
Bam HI
rrnBT1T2
Beta 2
Tac
pKKβ27
Sal I

FIG. 9

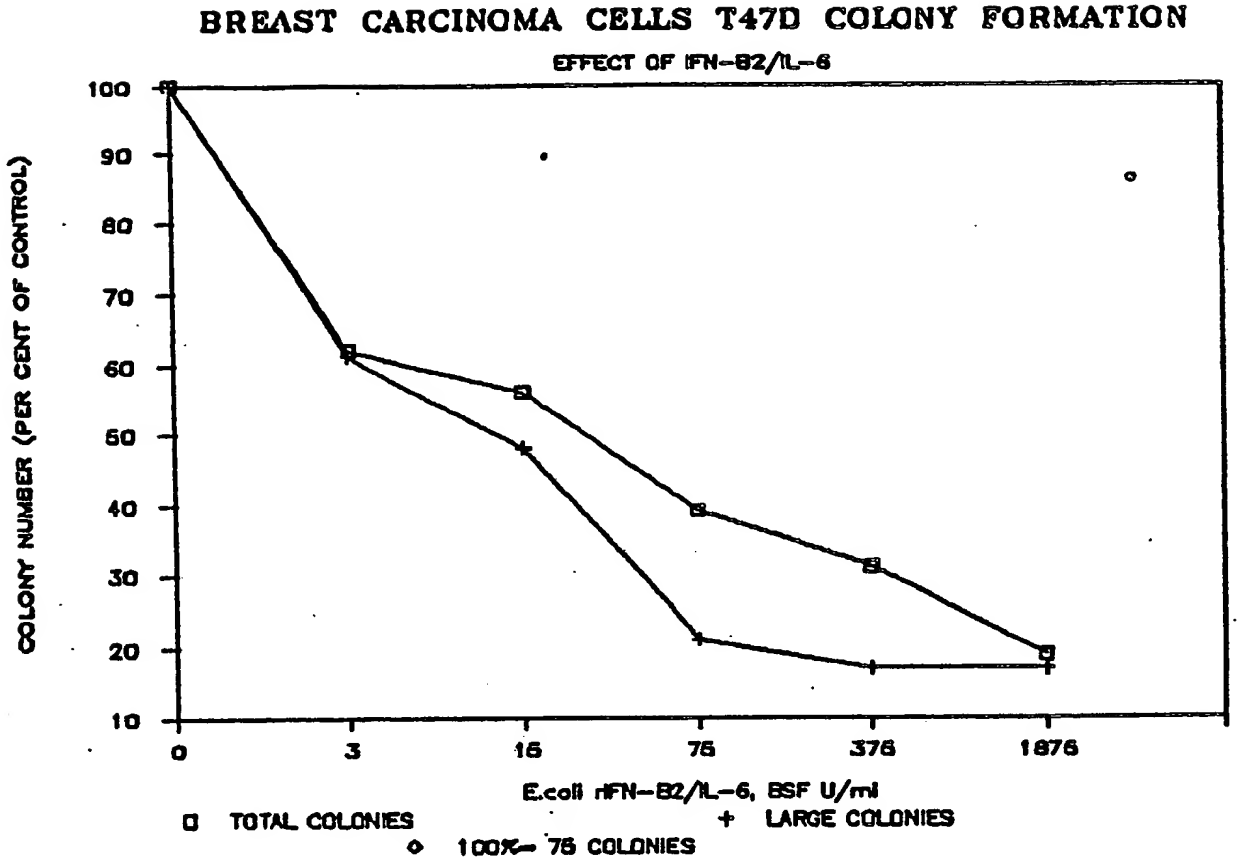
BREAST CARCINOMA CELLS T47D COLONY FORMATION
EFFECT OF IFN-B2/IL-6
COLONY NUMBER (PER CENT OF CONTROL)
100
90
80
70
60
50
40
30
20
10
0
3
16
76
376
1876
E.coli rIFN-B2/IL-6, BSF U/ml
TOTAL COLONIES
LARGE COLONIES
100%= 76 COLONIES

FIG. 11

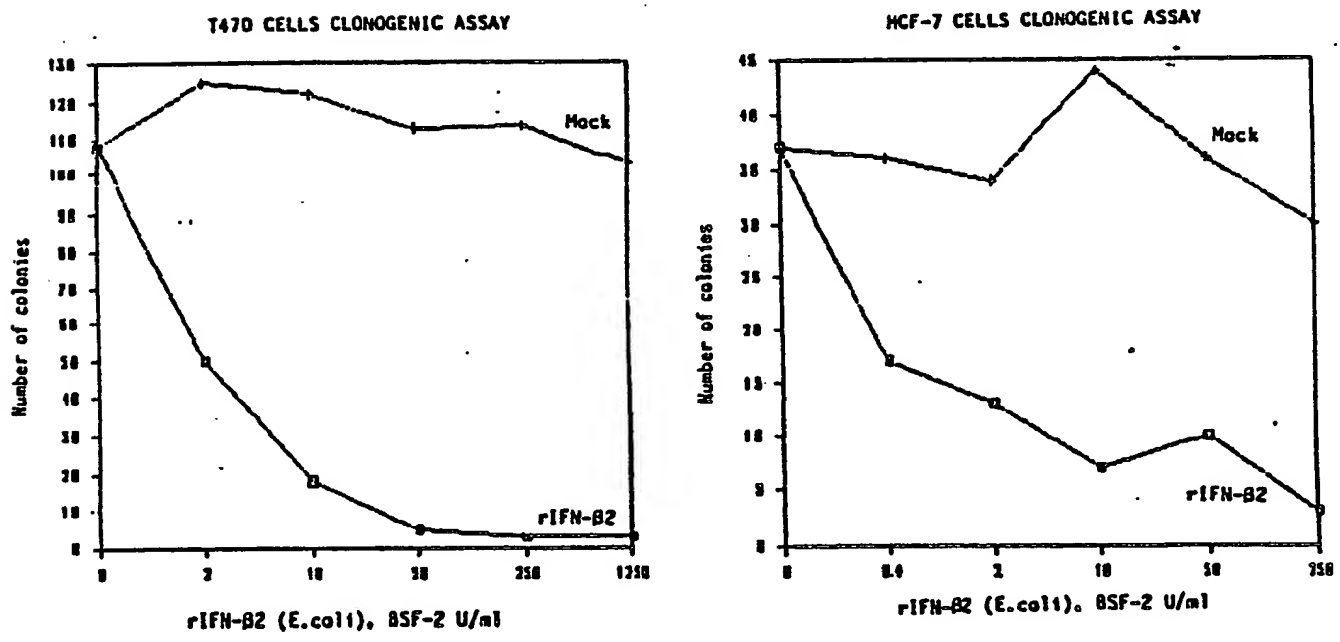
T47D CELLS CLONOGENIC ASSAY
Number of colonies
Mock
rIFN-B2
rIFN-B2 (E.coli), BSF-2 U/ml
HCF-7 CELLS CLONOGENIC ASSAY
Number of colonies
Mock
rIFN-B2
rIFN-B2 (E.coli), BSF-2 U/ml

FIG. 12

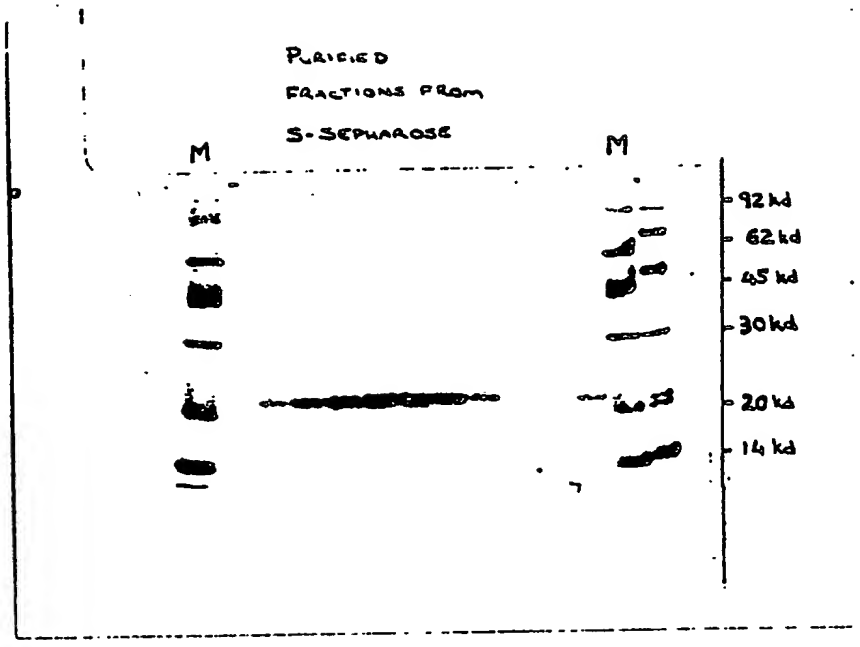

FIG. 10

DIFFERENTIATION OF MYELOLEUKEMIC M1 CELLS INDUCED BY IFN-β2/IL-6

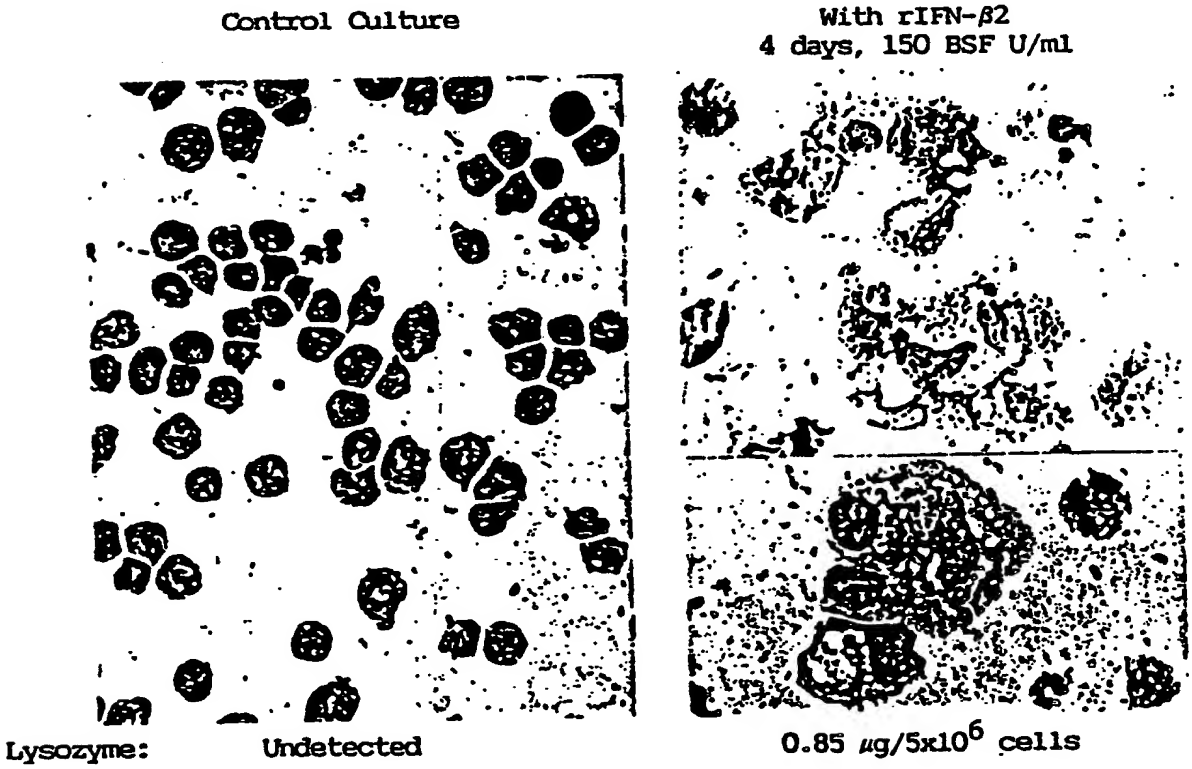

FIG. 13

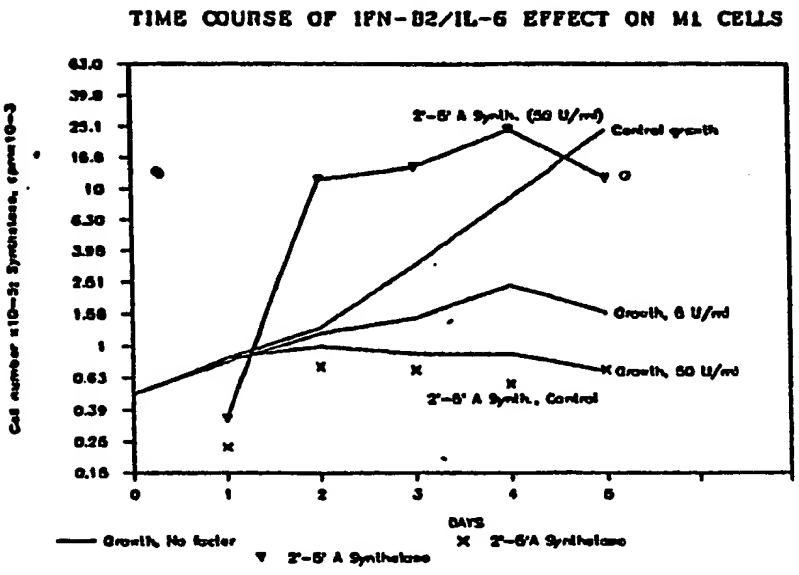
TIME COURSE OF IFN-B2/IL-6 EFFECT ON M1 CELLS
2'-5' A Synth. (50 U/ml)
Control growth
Growth, 6 U/ml
Growth, 50 U/ml
2'-5' A Synth., Control
DAYS
Growth, No factor
2'-5' A Synthetase
2'-5'A Synthetase

FIG. 14

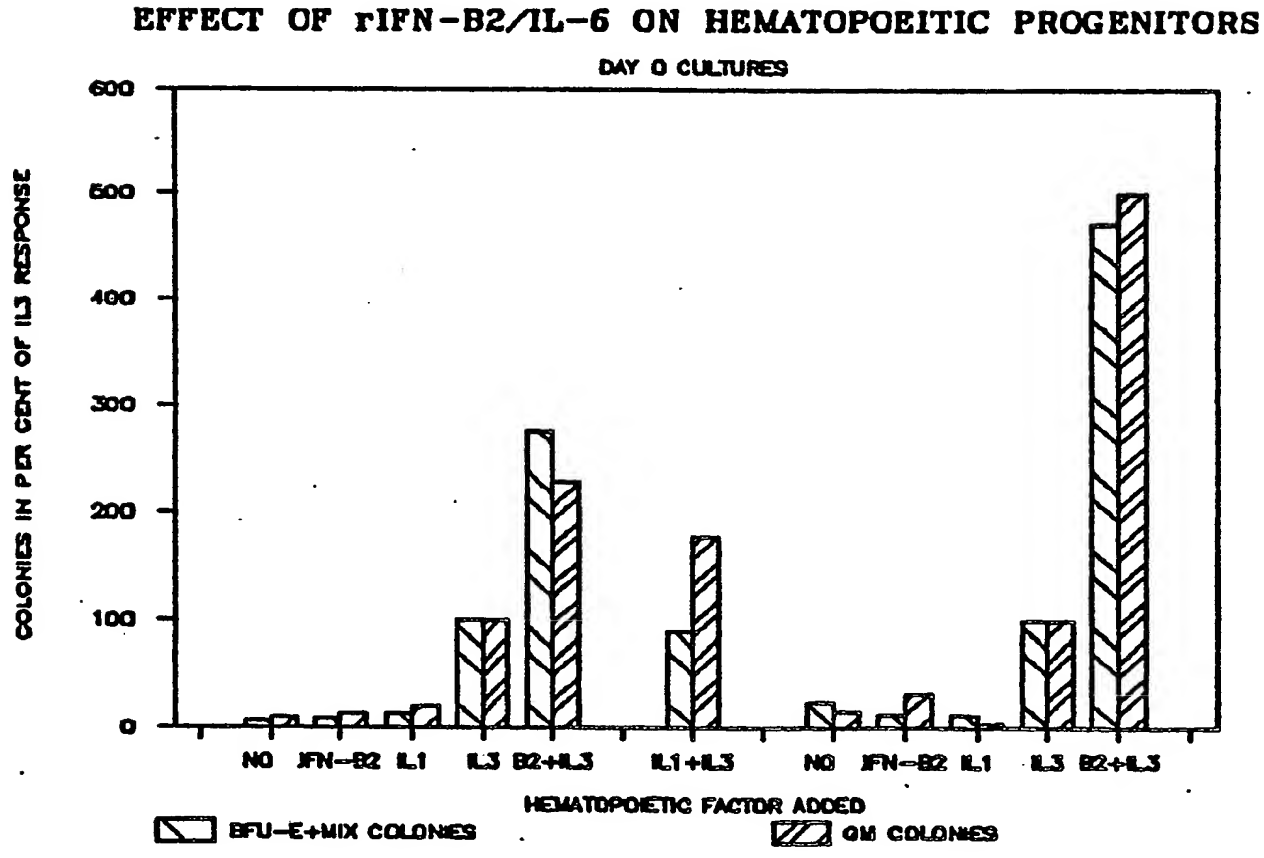
EFFECT OF rIFN-B2/IL-6 ON HEMATOPOEITIC PROGENITORS
DAY 0 CULTURES
COLONIES IN PER CENT OF IL3 RESPONSE
NO
IFN-B2
IL1
IL3
B2+IL3
IL1+IL3
HEMATOPOIETIC FACTOR ADDED
BFU-E+MIX COLONIES
GM COLONIES

FIG. 15